Ammonium Production in Cultures of *Bipolaris maydis* Race T: Evidence for Involvement of Asparaginase and NADP- and NAD-Glutamate Dehydrogenases[1]

T. W. BISCHOFF AND M. O. GARRAWAY, Department of Plant Pathology, The Ohio State University, Columbus, OH 43210

ABSTRACT. When *Bipolaris maydis* race T was incubated in a liquid medium (2 g/l glucose, 4 g/l L-asparagine, and mineral salts), ammonium and pH failed to increase after 48 h, but both increased after 72 h from 0.0 to 21.8 μmoles NH_4^+/ml, and from pH 5.8 to 7.9. Also at 72 h, glucose was no longer detected. When the activities of NADP-glutamate dehydrogenase (NADP-gdh), NAD-glutamate dehydrogenase (NAD-gdh) and L-asparaginase were assayed, NADP-gdh was high at 48 h and low at 72 h, and NAD-gdh was low at 48 h and high at 72 h. There were no differences in asparaginase activity after 48 or 72 h. To test the effects of cycloheximide on enzyme activity and ammonium accumulation, filter-sterilized (pore size 0.45 μm) cycloheximide was added at a rate of 10 μg/ml at 48 h and the assays were made at 72 and 96 h. Addition of cycloheximide inhibited dry weight increase, stopped removal of glucose from the culture medium, prevented pH increase, and blocked the transition from high NADP-gdh to high NAD-gdh activity. Only 10.0 μmoles/ml ammonium accumulated in cycloheximide-treated cultures in contrast to 21.8 μmoles/ml in the control, and asparaginase activity was unaffected. Thus, ammonium accumulation following glucose depletion may be related to the reduction of ammonium assimilated via NADP-gdh and the production of ammonium via asparaginase and NAD-gdh.

OHIO J. SCI. 86 (4): 186-189, 1986

INTRODUCTION

Several enzymes have been reported to be involved in ammonium production in fungi. Of these L-asparaginase (E. C. 3.5.1.1), NADP-glutamate dehydrogenase (E. C. 1.4.1.4), and NAD-glutamate dehydrogenase (E. C. 1.4.1.2) have been reported for several ascomycetous fungi (Arima et al. 1972, Imada et al. 1973, LéJohn 1971). Asparaginase has been isolated and characterized for *Fusarium tricinctum* (Scheetz et al. 1971), *Candida utilis* (Sakamoto et al. 1977a, 1977b) and *Saccharomyces cerevisiae* (Dunlop et al. 1980), and shown to catalyze the deamidation of asparagine to aspartate and ammonium. Both NADP-gdh and NAD-gdh have been implicated in ammonium regulation in fungi. The primary function of NADP-gdh is amination of α-ketoglutarate to form L-glutamate, whereas NAD-gdh deaminates L-glutamate to form α-ketoglutarate and ammonium. Under conditions where glucose and ammonium were present, NADP-gdh activity was high and NAD-gdh activity was low. In contrast, in the absence of glucose and in the presence of L-glutamate, NAD-gdh activity was high and NADP-gdh was low (Kapoor and Grover 1970, Kinghorn and Pateman 1974).

A previous study of *Bipolaris maydis*, the southern corn leaf blight pathogen, indicated that when the fungus was incubated with an L-asparagine-containing medium, ammonium accumulated (Bischoff and Garraway, 1985b). In that study, initial glucose concentration in the solid medium and time of incubation appeared to influence the level of ammonium observed. At six days, ammonium levels were higher with 2 g/l glucose than with 10 g/l; however, at 10 days there were no differences in ammonium levels with regard to glucose treatment. These observations were identical for both races and mating types of *B. maydis* (Bischoff and Garraway 1985b). In a preliminary study of *B. maydis* race T, it was found that ammonium accumulation occurred in an L-asparagine-containing liquid medium only after depletion of glucose (Bischoff and Garraway 1985a). The present study was carried out to determine if ammonium production in cultures of *B. maydis* race T could be related to the activities of asparaginase, NADP-gdh, and NAD-gdh. In addition, cycloheximide was added prior to glucose depletion to determine its effects on patterns of enzyme activity and ammonium accumulation.

MATERIALS AND METHODS

In this study, a liquid medium was used because the fungus grew rapidly, and the ammonium, pH and glucose levels in the culture fluid could be assayed easily. The basal medium consisted of 2.0 g D-glucose, 4.0 g L-asparagine, 1.5 g KH_2PO_4, 0.75 g $MgSO_4$, and 0.1 mg of the hydrated forms of $CuSO_4$, $Fe_2(SO_4)_3$, $MnSO_4$, and $ZnSO_4$ per liter of double-distilled water. Two g/l glucose were used because previous studies indicated that responses were similar between 2 and 10 g/l glucose (Bischoff and Garraway 1985b), and because it was difficult to get good agitation of mycelium and the medium using 10 g/l glucose. The initial pH was adjusted to 5.8 with 1 N KOH, and each compound was autoclaved (1.2 kg/cm^2 pressure for 20 min at 121°C) individually in solution. An aliquot of each solution was added to a 250-ml Erlenmeyer flask containing sterilized, double-distilled water. The final volume of the medium was 40 ml.

The isolate used in these studies was a single spore isolate of *Bipolaris maydis* race T (ATCC 36180) recovered from an ear of corn in Franklin County, Ohio in 1970. Flasks were seeded with three cores (4 mm diam) of mycelium and conidia from 10-day-old cultures grown on a glucose (10 g/l), L-asparagine (4 g/l), mineral salts agar medium. Cultures were incubated in darkness at 28 ± 2°C on reciprocal shakers (100/min) for 48, 72 or 96 h. After each incubation time, the culture fluid was analyzed for ammonium, pH, and glucose levels. The mycelium was used to determine the dry weight and NADP-gdh, NAD-gdh, and asparaginase activities. To test the effects of cycloheximide on enzyme activity and ammonium accumulation, an aliquot of filter-sterilized (pore size 0.45 μm) cycloheximide was added at a final concentration of 10 μg/ml following 48 h of incubation. Preliminary work indicated that cultures treated with this level of cycloheximide showed no further increase in protein levels (Bischoff unpublished data).

Following incubation, the pH of the culture medium was measured directly in the flask with a pH meter. To determine dry weight of the fungus, mycelium and the culture fluid were separated by suction

[1]Manuscript received 20 March 1986 and in revised form 25 July 1986 (#86-16).

filtration. Mycelium was deposited on a filter paper tare, taken to dryness in an oven at 100°C, and weighed.

Ammonium levels were determined as previously described (Bischoff and Garraway 1985b) by taking an aliquot of culture fluid and adding double-distilled water followed by Nessler's reagent. Absorbance of the solution was read at 450 nm after 15-20 min. Previous work has indicated that this is a reliable method for determining the quantity of ammonium in the medium (Bischoff and Garraway 1985b) and that autoclaved L-asparagine does not significantly contribute to ammonium levels.

Glucose levels were determined by Nelson's procedure for spectrophotometric determination of reducing sugars (Nelson 1944). An aliquot of the culture fluid was added along with Nelson-Somogyi reagent to an assay tube. The solution was incubated in boiling water for 20 min. After the tubes had cooled, arseno-molybdic color reagent was added followed by double-distilled water. After 15-20 min, absorbance was read at 540 nm. Reducing sugar present was determined by generation of a standard curve with known quantities of glucose. Paper chromatography of the culture fluid and detection of sugars with silver nitrate indicated that glucose was the only sugar present.

The NADP- and NAD-gdh activities were assayed with the procedure of Kinghorn and Pateman (Kinghorn and Pateman 1974). Frozen mycelium from three cultures of each treatment were crushed in a Carver press (4000 kg/cm^2). The extract and mycelium were collected, added to 5.0 ml phosphate buffer (0.05 M, pH 7.75), homogenized for 30 sec in a Polytron homogenizer to suspend the mycelium, and sonicated for 1 min in a Bronson sonicator. The mixture was centrifuged at 6800 *g* for 5 min, and the cell-free supernatant was used for the enzyme assay. Activity of NADP-gdh was determined by measuring the change in absorbance at 340 nm of a reaction mixture containing 0.4 ml of 0.4 M NH_4Cl, 0.2 ml of 0.2 M α-ketoglutarate, 0.2 ml of reduced NADP (2 mg/ml), and 2.0 ml of phosphate buffer (0.05 M, pH 7.75). A 0.2-ml aliquot of supernatant was added to initiate the reaction. The NAD-gdh activity was measured in a similar manner except 0.2 ml of reduced NAD (2 mg/ml) was used, and the phosphate buffer (0.05 M pH 8.0) contained 0.1 mM β-mercaptoethanol and 0.5 mM EDTA.

Asparaginase activity was not detected in the culture fluid after incubation with the fungus or in the supernatant after crushing the mycelium as described above for determination of the glutamate dehydrogenase activity. The mycelial pellet was able to release ammonium from L-asparagine; consequently, another method was used. Asparaginase activity could be detected in the cell-free supernatant after grinding the pellet with sand in phosphate buffer (0.05 M, pH 7.4) using a mortar and pestle and centrifuging again at 6800 *g* for 5 min. Asparaginase activity was assayed with the procedure described by Dunlop and Roon (Dunlop and Roon 1975). The assay system contained 2.0 ml of phosphate buffer (0.05 M, pH 7.4), 0.2 ml of 0.2 M α-ketoglutarate, 16.0 units of glutamate-oxaloacetate transaminase, 7.0 units malate dehydrogenase, and 0.2 ml of reduced NAD (2 mg/ml). Initially 0.4 ml of extract was added to determine the rate of NADH oxidation by recording absorbance at 340 nm. To determine L-asparaginase activity, 0.2 ml of 0.4 M L-asparagine was added and the rate of NADH oxidation determined.

The amount of protein was determined with the Lowry method (Lowry et al. 1951). Enzyme activity was expressed on the basis of μmoles coenzyme oxidized/mg protein/min.

Results reflect trends observed in three similar experiments. The data presented are representative of one of these experiments. Data are presented as means along with their 95% confidence interval. Significance ($P = 0.05$) was based on a Student's t-test.

RESULTS

Dry weight of *Bipolaris maydis* race T almost tripled from 48 to 72 h, increasing from 32.7 to 88.5 mg with no further increase from 72 to 96 h. Addition of cycloheximide at 48 h inhibited any further dry weight increase. Dry weight observed at 48 h was comparable to that observed in the cycloheximide treatment at 72 or 96 h (Fig. 1A). In the culture fluid after 48 h of incubation, about one-half of the initial 1875 μg/ml of glucose was still present. After 72 h no glucose was detected in the control. However, 420 and 595 μg/ml of glucose

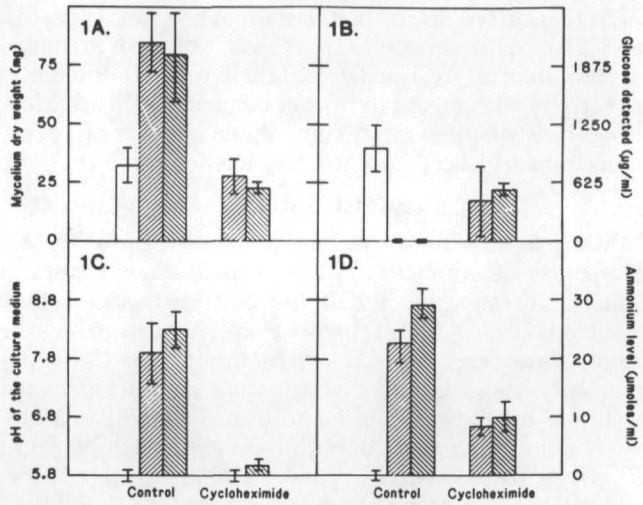

FIGURE 1. Dry weight of the mycelium and glucose, pH and ammonium levels in cultures of *Bipolaris maydis* race T after 48 (▭), 72 (▨), or 96 (▩) h of incubation on a glucose (2 g/l), L-asparagine (4 g/l), and mineral salts liquid medium. Cycloheximide (10 μg/ml) was added at 48 h of incubation. Means of four replications (vertical bars) and 95% confidence intervals are shown. Figure 1A, the dry weight of the mycelium; Figure 1B, glucose detected in the culture medium; Figure 1C, the pH of the culture medium; Figure 1D, ammonium levels in the culture medium.

were detected after 72 and 96 h, respectively, when cycloheximide was present (Fig. 1B).

The pH and ammonium levels in cultures of the fungus did not change during the first 48 h of incubation. At this time, no ammonium was detected and the pH was 5.8. However, by 72 h the pH increased to 7.9, and ammonium levels had increased to 21.8 μmoles/ml. Following 96 h of incubation, pH and ammonium levels were 8.3 and 29.2 μmoles/ml. Addition of cycloheximide inhibited the increases in pH seen between 48 and 72 h or 48 and 96 h; however, ammonium was produced in these cultures and averaged 8.7 and 10.0 μmoles/ml at 72 and 96 h, respectively (Figs. 1C and 1D).

The activity of NADP-gdh was high after 48 h and low after 72 or 96 h of incubation (Fig. 2A). In contrast, NAD-gdh activity was low after 48 h and high after 72 h (Fig. 2B). However, the activity of this enzyme dropped dramatically between 72 and 96 h (Fig. 2B). Addition of cycloheximide at 48 h blocked the change from high

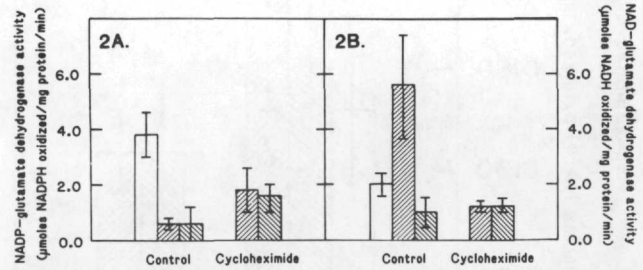

FIGURE 2. NADP-glutamate dehydrogenase and NAD-glutamate dehydrogenase activities in cell-free extracts of *Bipolaris maydis* race T after 48 (▭), 72 (▨), or 96 (▩) h of incubation on a glucose (2 g/l), L-asparagine (4 g/l), and mineral salts liquid medium. Cycloheximide (10 μg/ml) was added after 48 h of incubation. Means of three replications (vertical bars) and 95% confidence intervals are shown. Figure 2A, NADP-glutamate dehydrogenase activity; Figure 2B, NAD-glutamate dehydrogenase activity.

NADP-gdh to high NAD-gdh activities (Figs 2A and 2B). Asparaginase activity was detected in fungal mycelium of all treatments, and there were no differences in activity with regard to time of incubation. In addition, levels of asparaginase were comparable whether or not the mycelium had been exposed to cycloheximide (Fig. 3).

DISCUSSION

When *Bipolaris maydis* race T was incubated in a glucose, L-asparagine, mineral salts liquid medium, increases in ammonium and the pH of the culture medium were detected after 72 h of incubation. At this time the ammonium level was 21.8 μmoles/ml, and the pH was 7.9. These levels of ammonium and pH detected in liquid media were similar to those reported in a previous solid media study of both races and mating types of *B. maydis* (Bischoff and Garraway 1985b). In that study, after 10 days ammonium levels averaged 17.5 μmoles/ml, and the pH was 8.2. Thus, it appears that ammonium production and the pH changes are similar in solid and liquid media.

Production of ammonium in cultures of *B. maydis* race T coincided with the depletion of glucose from the culture medium. Previous reports with *Neurospora crassa* (Kapoor and Grover 1970) and *Aspergillus nidulans* (Kinghorn and Pateman 1974) indicated that the aminating activity of NADP-gdh was high in the presence of glucose and low in its absence, whereas the deaminating activity of NAD-gdh was low in the presence of glucose and high in its absence. When NADP-gdh, NAD-gdh, and asparaginase activities were monitored in cultures of *B. maydis* race T, there was no change in asparaginase activity at any incubation time. In contrast, there was a shift from high NADP-gdh activity at 48 h, when glucose was detectable in the medium, to high NAD-gdh activity at 72 h, when glucose was no longer detectable. Cycloheximide was added at 48 h to examine the effect of a protein synthesis inhibitor on ammonium production. Cycloheximide inhibited dry weight increase, pH increase, and the removal of glucose from the culture medium. However, ammonium levels were intermediate between the control at 48 and 72 h. When enzyme activities were compared, cycloheximide had no effect on asparaginase activity. However, it blocked the change from high NADP-gdh to high NAD-gdh observed between 48 and 72 h. Further research is needed to determine whether depletion of glucose is necessary for the change in glutamate dehydrogenase activity, or whether glucose levels must simply drop below a threshold level.

Although *B. maydis* race T had the capacity to produce ammonium from L-asparagine by asparaginase after 48 h, no ammonium accumulated in the culture medium. However, ammonium did accumulate in these cultures after 72 h. One explanation for this might be that the fungus had not synthesized enough asparaginase to lead to a build up of ammonium. Another hypothesis is that the fungus does not degrade L-asparagine until glucose is consumed. Alternatively, the ammonium produced by the asparaginase may be assimilated via NADP-gdh activity as reported for *N. crassa* (Dantzig et al. 1979). The correlation between glucose depletion and ammonium accumulation in cultures of *B. maydis* race T may be due to a switch from ammonium assimilation mediated by NADP-gdh to ammonium production caused by a reduction in the activity of this enzyme, an increase in NAD-gdh activity, and continued asparaginase activity.

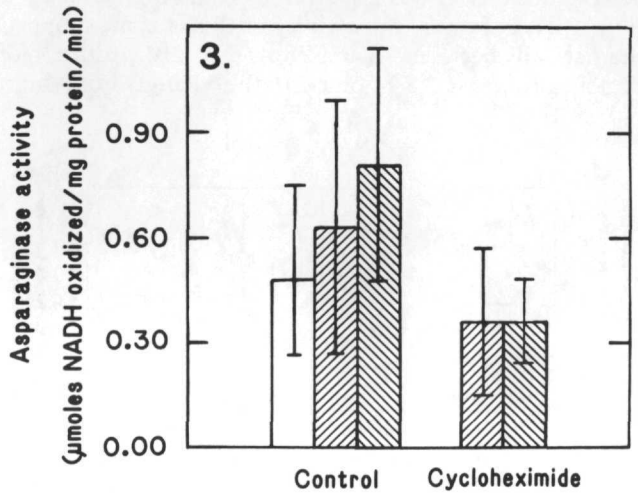

FIGURE 3. Asparaginase activity in cell-free extracts of *Bipolaris maydis* race T after 48 (▭), 72 (▨), or 96 (▩) h of incubation on a glucose (2 g/l), L-asparagine (4 g/l), and mineral salts liquid medium. Cycloheximide (10 μg/ml) was added after 48 h of incubation. Means of three replications (vertical bars) and 95% confidence intervals are shown.

ACKNOWLEDGMENTS. We thank Dr. I. W. Deep and Dr. R. M. Riedel for reviewing this manuscript. Salaries and research support provided by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Submitted as Journal Article No. 173-85 from the Ohio Agricultural Research and Development Center.

LITERATURE CITED

Arima, K., T. Sakamoto, C. Araki, and G. Tamura 1972 Production of extracellular L-asparaginase by micro-organisms. Agric. Biol. Chem. 36: 356-361.

Bischoff, T. W. and M. O. Garraway 1985a Changes in amount of ammonium released from L-asparagine by *Bipolaris maydis* race T. Ohio J. Sci. 85: 14 (abstract).

——— and ——— 1985b Ammonium production by races and mating types of *Bipolaris maydis* and its relationship to mycelium dry weight, sporulation and pH of the culture medium. Ohio J. Sci. 85: 155-158.

Dantzig, A. H., F. L. Wiegmann, and A. Nason 1979 Regulation of glutamate dehydrogenases in nit-2 and am mutants of *Neurospora crassa*. J. Bacteriol. 137: 1333-1339.

Dunlop, P. C., G. M. Meyer, and R. J. Roon 1980 Reactions of asparaginase II of *Saccharomyces cerevisiae*. J. Biol. Chem. 255: 1542-1546.

——— and R. J. Roon 1975 L-asparaginase of *Saccharomyces cerevisiae:* An extracellular enzyme. J. Bacteriol. 122: 1017-1024.

Imada, A., S. Igarasi, K. Nakahama, and M. Isono 1973 Asparaginase and glutaminase activity of micro-organisms. J. Gen. Microbiol. 76: 85-99.

Kapoor, M. and A. K. Grover 1970 Catabolite-controlled repression of glutamate dehydrogenases of *Neurospora crassa*. Can. J. Microbiol. 16: 33-40.

Kinghorn, J. R. and J. A. Pateman 1974 The effect of the carbon source on ammonium regulation in *Aspergillus nidulans*. Molec. Gen. Genet. 128: 95-98.

LéJohn, H. B. 1971 Enzyme regulation, lysine pathways and cell wall structure as indicators of major lines of evolution in fungi. Nature (Lond.) 231: 164-168.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall 1951 Protein measurement with Folin Phenol reagent. J. Biol. Chem. 193: 265-275.

Nelson, N. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

Sakamoto, T., C. Araki, T. Beppu, and K. Arima 1977a Partial purification and some properties of extracellular asparaginase from *Candida utilis*. Agric. Biol. Chem. 41: 1359-1364.

———, ———, ———, and ——— 1977b Extracellular asparaginase from *Candida utilis*, its properties as glycoprotein and antitumor activities. Agric. Biol. Chem. 41: 1365-1371.

Scheetz, R. W., H. A. Whelan, and J. C. Wriston 1971 Purification and properties of an L-asparaginase from *Fusarium tricinctum*. Arch. Biochem. Biophys. 142: 184-189.